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IDENTIFICATION, SEQUENCE AND EXPRESSION OF A CRUSTACEAN CARDIOACTIVE PEPTIDE (CCAP) GENE IN THE MOTH *MANDUCA SEXTA*

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Summary

The crustacean cardioactive peptide (CCAP) gene was isolated from the tobacco hawkmoth *Manduca sexta*. The gene has an open reading frame of 125 amino acid residues containing a single, complete copy of CCAP. Analysis of the gene structure revealed three introns interrupting the coding region. A comparison of the *M. sexta* CCAP gene with the *Drosophila melanogaster* genome database reveals significant similarities in sequence and gene structure.

The spatial and temporal expression patterns of the CCAP gene in the *M. sexta* central nervous system were determined in all major post-embryonic stages using *in situ* hybridization techniques. The CCAP gene is expressed in a total of 116 neurons in the post-embryonic *M. sexta* central nervous system. Nine pairs of cells are observed in the brain, 4.5 pairs in the subesophageal ganglion, three pairs in each thoracic ganglion (T1–T3), three pairs in the first abdominal ganglion (A1), five pairs each in the second to sixth abdominal ganglia (A2–A6) and 7.5 pairs in the terminal ganglion. The CCAP gene is expressed in every ganglion in each post-embryonic stage, except in the thoracic ganglia of first- and second-instar larvae. The number of cells expressing the CCAP gene varies during post-embryonic life, starting at 52 cells in the first instar and reaching a maximum of 116 shortly after pupation. One set of thoracic neurons expressing CCAP mRNA shows unusual variability in expression levels immediately prior to larval ecdysis. Using previously published CCAP immunocytochemical data, it was determined that 91 of 95 CCAP-immunopositive neurons in the *M. sexta* central nervous system also express the *M. sexta* CCAP gene, indicating that there is likely to be only a single CCAP gene in *M. sexta*.

Key words: crustacean cardioactive peptide, cardioacceleratory peptide 2a, neuropeptide, insect peptide, gene, *Manduca sexta*, *Drosophila melanogaster*.

Introduction

Peptide signaling factors, whether found in the nervous system or peripheral tissues, are ubiquitously distributed throughout the animal kingdom and have been demonstrated to act as neurotransmitters, modulators and hormones in important life processes. The functional significance of peptides is highlighted by the fact that their absence often leads to severe handicap or even the demise of the animal. One such peptide is the crustacean cardioactive peptide (CCAP), which plays a variety of well-elucidated roles in insects and other invertebrates.

CCAP was first studied in insects in the moth *Manduca sexta*. It was originally known as cardioacceleratory peptide 2a (CAP2a), one of several cardioacceleratory peptides isolated from the central nervous system (CNS) of *M. sexta* (Tublitz and Truman, 1985a; Tublitz and Truman, 1985b; Tublitz and Truman, 1985c; Tublitz and Truman, 1985d; Tublitz et al., 1991; Tublitz and Loi, 1993). When the primary structure of CAP2a was elucidated in 1992 (PFCNAFTGCamide; Cheung et al., 1992; Lehman et al., 1993), it was determined to be structurally identical to CCAP, a crustacean peptide (Stangier et al., 1987). CCAP has several well-described functions in *M. sexta*. In adults, it acts in an excitatory fashion on the heart during adult wing inflation (Tublitz and Truman, 1985b) and adult flight (Tublitz, 1989). In larvae, CCAP is thought to increase the frequency of gut contractions on two separate occasions, first during embryonic development (Broadie et al., 1990) and again to empty the gut at the beginning of metamorphosis (Tublitz et al., 1992). CCAP also causes an increase in fluid secretion by the Malpighian tubules in pharate adult *M. sexta* (N. J. Tublitz and S. H. P. Maddrell, unpublished observations). In addition to its effects on peripheral tissues, CCAP acts centrally as the proximal trigger of ecdysis behavior at the larval–pupal molt in *M. sexta* (Gammie and Truman, 1997).

CCAP also plays a variety of functional roles across a broad range of arthropods and in several other invertebrate phyla. In insects, CCAP triggers an increase in heart rate in *Drosophila melanogaster* and *Manduca sexta* (Tublitz et al., 1994; Tublitz

and Evans, 1986), modulates oviduct contractions in *Locusta migratoria* (Donini et al., 2001) and alters hindgut activity during wandering in *Manduca sexta* (Tublitz et al., 1992). CCAP is also found in crustaceans, triggering ecdysis behavior in the crab *Carcinus maenas* (Philippen et al., 2000) and modulating stomatogastric motor patterns in the lobster *Homarus americanus* (Richards and Marder, 2000). CCAP may also play a modulatory and/or hormonal role in gastropod molluscs on the basis of the presence of CCAP immunoreactivity in neurosecretory cells located in the CNS of *Lymnaea stagnalis* and *Helix aspersa* (Hernadi and Agricola, 2000).

To date, little is known about CCAP gene structure and gene expression in any organism. The aim of this study was to begin to address this issue in the moth *Manduca sexta*, in which much of the early work on CCAP was performed. Using standard molecular methods, we have isolated and sequenced a CCAP-coding gene in *M. sexta*. We also present *in situ* hybridization data on the spatial and temporal expression patterns of this gene in all post-embryonic stages.

Materials and methods

Polymerase chain reaction (PCR) screening of a Manduca sexta *cDNA library*

Material used in PCR

PCR screening was performed on a *Manduca sexta* λ Zap cDNA library produced from whole nerve cords of day 3 fifth instars by Stratagene Corp. A DNA template for PCR was obtained by making a 50 ml liquid culture from 100 µl of the unamplified *Manduca sexta* λ Zap cDNA library using the protocol described by Sambrook et al. (Sambrook et al., 1989). Two sets of primers were used for the initial screen. The first set consisted of a degenerate oligonucleotide (CCAP sense; Gibco BRL) and a universal T7 primer (Promega). The second set of primers consisted of a degenerate CCAP antisense oligonucleotide (CCAP antisense) and a universal T3 primer. The CCAP sense primer was constructed using the amino acid sequence of CCAP (Cheung et al., 1992) and was built to recognize the sense strand. The sequence of this primer was 5′-AA(C/T)GC(I)TT(C/T)AC(I)GG(I)TG(C/T)GG. The CCAP antisense primer was constructed to recognize the antisense strand of CCAP and had the sequence 5′-CCCCGTGAACGC-GTT(A/G)CA(A/G)AA(G/A/T/C). Inosine (I) was inserted in several fully redundant positions. T7 and T3 are universal primers, and their binding sites are located on the arms of the λ Zap vector used to construct the library. Taq polymerase, dNTPs, $MgCl_2$ and 10× polymerase chain reaction (PCR) buffer (500 mmol l^{-1} KCl, 100 mmol l^{-1} Tris-HCl, pH 9, and 1 % Triton X-100) were purchased from Promega.

The following were used for each 50 µl PCR sample: 4 µl of 10 mmol l^{-1} dNTPs, 5 µl of 10× PCR buffer, 8 µl of 25 mmol l^{-1} $MgCl_2$, 1.5 units of Taq polymerase, 150 pmol of the degenerate oligonucleotide primer, 22.8 pmol of the vector primer T7 and 3 µl of reamplified cDNA template together with sterile water to bring the reaction volume to 50 µl. PCR was performed in a Peltier PTC-200 Thermocycler from MJ Research using the following program: 2 min at 94 °C, 10 min at 80 °C, 2 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C, with the last three steps repeated for 35 cycles. The reaction was then held at 72 °C for 15 min, terminating at a holding temperature of 4 °C. Taq polymerase was added during the 10 min holding step at 80 °C. PCR products were separated by gel electrophoresis in 5 % polyacrylamide gels. DNA was visualized under ultraviolet light after a 5 min incubation with 15 µl of ethidium bromide in 100 µl of double-distilled water followed by a brief rinse in double-distilled water.

Subcloning and transformation

PCR products from the above PCR reaction were run on a 1 % Sea Plaque low-melt agarose gel (FMC Bioproducts) in E buffer (48 g of Trizma base, 7.4 g of disodium EDTA dissolved in 600 ml of water, pH adjusted to pH 8 with acetic acid). The bands of interest were excised, and the DNA within the gel was cloned into a PCR 2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen) following the procedure specified by the manufacturer.

DNA sequencing

Cloned PCR products were purified using a Wizard SV miniprep kit (Promega). A restriction enzyme digest with *Eco*RI (Promega) was performed to confirm that an insert of the correct size was present in each sample. Purified products were sequenced at the University of Oregon DNA sequencing facility with the vector primers M13 forward and reverse (Invitrogen).

To check whether the sequences obtained using CCAP sense/T7 and CCAP antisense/T3 primer sets were correct, PCR was performed using another set of primers, CCAPT7 (sense) and CCAPT3 (antisense). The CCAPT7 sense primer was made to recognize a portion of the sequence downstream of the putative CCAP coding region and had the sequence 5′-CCTAATTAGTACCTACTGTTG. The CCAPT3 antisense primer was constructed from a sequence upstream of a putative start codon of the putative sequence. Its sequence was 5′-CGGCTGGAAACAATGACTGTA. The product from this PCR was also subcloned and sequenced.

Isolation of the CCAP gene using rapid amplification of mRNA ends (RACE)

Reverse transcriptase PCR (RT-PCR) was also performed using degenerate primers designed on the CCAP sequence. Messenger RNA was isolated from dissected *M. sexta* CNS using a Dynabeads mRNA direct kit (Dynal, Lake Success, NY, USA). First-strand cDNA was synthesized by 1 unit of Superscript RT (Gibco BRL Life Technologies, Gaithersburg, MD, USA) for 1 h at 37 °C with 10 pmol of adaptor-oligo dT primer (5′-GACTCGAGTCGACATCGAT+17T). The 3′ rapid amplification of mRNA end (RACE) was composed of two consecutive rounds of PCR reactions using two partially overlapping degenerate primers designed on the CCAP peptide sequence. The first round of PCR was performed with primers CCAPf1 [5′-CCITT(C/T)TG(C/T)AA(C/T)GC(A/G/

C/T)TT(C/T)AC] and adaptor-oligo dT primer. Then, the second round of PCR was performed using nested primer CCAPf2 [5′-AA(C/T)GC(A/G/C/T)TT(C/T)AC(A/G/C/T)G-G(A/G/C/T)TG(C/T)GG] and adaptor primer (5′-GACTCGA-GTCGACATCGAT) using 1 μl of the first-round PCR product as template. After we had obtained the sequence for the 3′ side of the CCAP cDNA, we designed two reverse primers for 5′-RACE: CCAPr1 (5′-CAGCACTGGCTTCCTGGATG) and CCAPr2 (5′-GAATGTAGGCTTCCTTCTGC). Two rounds of nested PCR were performed using adaptor-oligo dG primer (5′-GACTCGAGTCGACATCGAT+17G), adaptor primer (sequence as above) and reverse primers.

The genomic structure of the open reading frame was analyzed by PCR using additional primers and genomic DNA as templates. These are the primers CCAPf3 (5′-TTCACACA-CCACCAGTCCAG), CCAPf4 (5′-GCTTGCTGCTTCTCGT-CGCCT), CCAPr3 (5′-TGCGACCGCAACCCGTGAAC) and CCAPr4 (5′-TTGAACTCACCCGTGAACGC).

The CCAP-coding transcript in *D. melanogaster* gene CG4910 was confirmed by RT-PCR 5′- and 3′-RACE using the primers DmCCAPf2 (5′-CACACTCCTGCAATGAGA-ACG), DmCCAPf1 (5′-CGTATCCCTCGTATCCGCCAT), DmCCAPr1 (5′-CATGATTTGCTTTTGAACATT) and DmCCAPr2 (5′-CGGAATGGCGGATACGAGG). Sub-cloning, transformation and DNA sequencing procedures were identical to those described above.

In situ *hybridization of the CCAP probe on the* Manduca sexta *central nervous system*

Experimental animals

Manduca sexta L. were raised at the University of Oregon and staged as previously described (Loi and Tublitz, 1993). The *in situ* hybridization experiments in this paper utilized animals from every major post-embryonic stage. All stages were raised in an environmentally controlled chamber with a 17 h:7 h L:D photoperiod and a superimposed thermal period (27 °C during the light cycle and 25 °C during the dark cycle) to synchronize development. Animals were staged on the basis of stage-specific developmental and/or behavioral events (Truman and Riddiford, 1974). D0 is designated as the day of the larval–larval molt; W0 is the day of the onset of wandering; P0 is the day of the larval–pupal molt. Other stage-specific developmental and/or behavioral markers used in this study include the head capsule slippage stage (HCP; inclusive of fluid-filled and air-filled head capsules) and P10 (pupal day 10, the day before the full development of the dorsal pad on the nerve cord). On the day of adult emergence, animals not yet emerged were designated as ‘pharate adults’; after emergence, adults were designated as adult day 0 (A0).

In situ *hybridization*

The DNA template for making the *in situ* hybridization probe was the purified DNA from the subcloned PCR product of the CCAPT3 and CCAPT7 primers, which contained the middle coding portion of the CCAP gene. The probes for the *in situ* experiments were prepared with a non-radioactive RNA labeling kit (DIG/Genius 4 RNA kit; Boehringer Mannheim) following the instructions provided by the manufacturer.

Animals were anesthetized by immersion in iced water until they were limp and unresponsive. The entire CNS, including the brain, was surgically removed from animals of various stages of development and placed into fixative (4 % paraformaldehyde in phosphate-buffered saline; PFA) within 30 min. Pipes saline (6.5 mmol l^{-1} $NaCl_2$, 28.5 mmol l^{-1} KCl, 16.2 mmol l^{-1} $MgCl_2{\cdot}6H_2O$, 5.6 mmol l^{-1} $CaCl_2$, 5 mmol l^{-1} Pipes; pH 6.7) was used in all dissections and rinses. Isolated nerve cords were fixed overnight. After 16–24 h, the fixed nerve cords were incubated in three ascending concentrations of methanol (33 %, 66 % and 100 %) for 10 min each and stored in 100 % methanol for 1–3 days. While in 100 % methanol, nerve cords were either pinned in Sylgard-lined culture dishes (20 mm diameter) or placed in 1.5 ml micro centrifuge tubes. Nerve cords from third-instar and older animals received an additional step prior to hybridization. They were pre-treated with proteinase K (Pro K) as follows: after 1–3 days of incubation in methanol, the nerve cords were washed with three ascending concentrations (33 %, 66 %, 3× 100 %) of phosphate-buffered saline with 0.1 % Tween20 (pH 7.4; PBST) in methanol for 3 min at each concentration. After the last wash, the PBST was replaced with 20 μg ml^{-1} Pro K (Sigma) for 2 min. Adult nerve cords were treated with a higher concentration of Pro K (200 μg ml^{-1}) for 10 min. After Pro K treatment, nerve cords were rinsed briefly three times in PBST and then twice for 5 min in PBST before post-fixing for 1 h in 4 % PFA.

Every CNS received the following treatment regardless of its developmental stage. After removal from methanol (first and second instars) or 4 % PFA (third instar to adult), the nerve cords were rinsed twice for 5 min in PBST, then pre-hybridized at 65 °C for 1 h in pre-hybridization solution [50 % formamide, 5× sodium citrate buffer (SSC), 500 μg ml^{-1} total RNA, 50 ng ml^{-1} heparin, 0.1 % Tween 20, 9.2 mmol l^{-1} citric acid]. The pre-hybridization solution was replaced with hybridization solution containing 1 μl ml^{-1} of probe pre-warmed to 65 °C, and nerve cords were incubated overnight.

After the overnight incubation, nerve cords underwent two series of washes. The first series of washes was with ascending concentrations of 2× SSC/pre-hybrization solution (33 %, 66 %, 100 %) at 65 °C (two washes per concentration, 2 min followed by 10 min) and ending with two washes of 0.2× SSC (10 min each). The second series of washes was with an ascending concentration of PBST/0.2× SSC (33 %, 66 %, 100 %; 5 min per wash) at room temperature (21 °C). After these two series of washes, the nerve cords were washed for another 5 min with PBST before incubation with blocking solution (1× PBS, 1 % bovine serum albumin, 0.1 % Tween 20). After 1 h, the blocking solution was replaced with an anti-digoxigenin antibody conjugated to alkaline phosphatase (1:5000; diluted in 0.1× blocking solution) and incubated overnight. Following the overnight primary antibody incubation, nerve cords were washed in six washes of PBST (5 min, 10 min, 2× 15 min and 2× 30 min). Thereafter, nerve cords were incubated with color-

developing buffer (100 mmol l^{-1} Tris, pH 9.5, 50 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} $NaCl_2$, 0.1 % Tween 20) for 10 min before replacing the buffer with the color developer (color-developing buffer plus 450 µg ml^{-1} 4-Nitroblue Tetrazolium chloride plus 175 µg ml^{-1} 5-bromo-4-chloro-3-indolyl-phosphate). This color-developing buffer was used for most experiments except for the last two trials. In one trial, 10 % (w/v) polyvinyl alcohol [PVA, 70–100 kDa (Sigma) dissolved at 90 °C] and 1 mmol l^{-1} levanisole (a potent inhibitor of lysosomal phophatases) were used to enhance color development. Levanisole and $MgCl_2$ were added after the Tris–NaCl–PVA had cooled. In another trial, only PVA was used in the color-developing buffer.

The reaction was allowed to proceed for 2–3 h for first-, second-, P10 and some third-instar animals or for 16–20 h for the other stages of development. After the color had precipitated, the reaction was stopped by washing the nerve cords in double-distilled water. Nerve cords were adhered onto poly-L-lysine (Sigma)-coated coverslips and dehydrated in an ascending ethanol series, 1× 70 % (10 min), 2× 90 % (5 min and 10 min), 2× 100 % (5 min and 10 min), and then cleared in 2× xylene (5 min and 10 min). Incubation times in 100 % ethanol and xylene were occasionally increased to optimize signal-to-background ratio. Permount was used for mounting a top coverslip onto the poly-L-lysine-coated coverslips.

Results

Isolation and primary sequence of a CCAP gene in Manduca sexta

As a first step in obtaining a CCAP gene, PCR was performed with two different sets of primers: CCAP antisense with T3, and CCAP sense with T7. Primer construction and sequence are described in detail in the Materials and methods section. The two primer sets produced two PCR products, 200 base pairs (bp) and 700 bp, respectively, which were subcloned and sequenced. The sequence of the 200 bp product contained part of the 3′ end of the λ Zap vector sequence followed by a methionine or start codon and the CCAP antisense primer sequence. The 700 bp product yielded a sequence that started with part of the CCAP sense primer sequence, a glycine codon and an RK coding for a C-terminal post-translational cleavage site. The sequence also contained a putative stop codon 204 bp downstream from the CCAP sequence. The two sequences overlapped at the peptide-coding region, the region used to design the initial two sets of degenerate PCR primers. To confirm that the sequence actually overlapped at the peptide-coding region, two other primers were generated using the preliminary sequences described above. The results of the PCR using these two new primers produced a product whose sequence perfectly matched that of the first two products. An independently performed RT-PCR yielded an identical sequence with slightly longer 5′ and 3′ ends.

The full sequence of the RT-PCR products, as shown in Fig. 1, contains 1135 nucleotides. There are 66 untranslated base pairs from the beginning of the sequence to the putative start initiation site. The single open reading frame is 375

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                                                                gattca      -6

    cacaccaccagtccaggcaaacgtgtttttaaaatctgacgtgatcaacggctggaaaca      -1

    ATGACTGTATCCCGAGTGTGCTTGCTGCTTCTCGTCGCCTTGGTGTACTTGGACTGCTGT      60
    M  T  V  S  R  V  C  L  L  L  L  V  A  L  V  Y  L  D  C  C
                  \/intron1 (243bp)
    TATGCAGCTTCGATTCCAAGAAACTTCGATCCCCGACTCAGTGAAGAAATTGTAATGGCT      120
    Y  A  A  S  I  P  R  N  F  D  P  R  L  S  E  E  I  V  M  A
                                                \/intron2 (229bp)
    CCCAAGAAGAGACCATTCTGCAACGCGTTCACGGGTTGCGGTCGCAAGCGCTCTCAGGGA      180
    P  K  K  R  P  F  C  N  A  F  T  G  C  G  R  K  R  S  Q  G
                                                \/intron3 (543bp)
    CCTCCCGGAATGCCTGCACAAGACTTGCGCACAAAACAGTACCTTGATGAAGAAGCTCTT      240
    P  P  G  M  P  A  Q  D  L  R  T  K  Q  Y  L  D  E  E  A  L

    GGGTCGATCCTCGACTCTGAATCGGCCATAGATGAACTCTCGCGCCAAATCCTCTCAGAG      300
    G  S  I  L  D  S  E  S  A  I  D  E  L  S  R  Q  I  L  S  E

    GCGAAACTCTGGGAAGCCATCCAGGAAGCCAGTGCTGAGATCGCCAGGCGGAAGCAGAAG      360
    A  K  L  W  E  A  I  Q  E  A  S  A  E  I  A  R  R  K  Q  K

    GAAGCCTACATTCAGTAAtgcaatgaactcacgatttaaccttatgtatagaccgtagcg      420
    E  A  Y  I  Q  *

    tatgtagcagaatgtattaagttaggacgttgccgtgattagcgccatattggaatttat      480
    gaaatgggatataagtacttagttgacaaatatacgaatatttcgcttctttaacacaat      540
    cagataaagcgtactgtgtttatataaatgtaatttttattgaacattgaggtacatattt      600
    tggaatgtgaatgaagatttcaaaacgcaataaaattatcaacagtaggactaattaggt      660
    tctactcatttttaaattgttatggaaataaatacgctaaagtattgagatttttttat      720
    taaaatagactagtaaatatctaagaggtgattttgtcaagaagctatgcttagctttct      780
    gtatgtgtagtttcaaattgtcttctaactataatatatattataacatcgaattatgat      840
    agttagtgtagttggtttctttttacattggttctataaaattacaaaatgattctattt      900
    ttttttgtattcaatacacataatataagtatttactgactataaataaaaattaaattg      960
    cattaattatcaagttcttttatattacaaataattctttgtagaaccaatatcaaaagg     1020
    aggacatcaatccacaaacaccgattttgttatttgaaaatgcacatacaagcttgatt     1080
    ggcgaatttcaatgtagcctaaaggtttaaataaaacgatctatctgt polyA          1128

Intron 1: 243bp

GTAATGTTATACTTTTACCTTTGAAATTGACATGAAGATTTCACGGAGTGATTAATGTAGGTTGCCTCATGACGT
TTCCCTATACTATCAAGGCTAGCAATAATTGACCAAGAATACTGTGTCTAAATTTGGGAATTATTTAATTTTTGC
ATATTATAAAACAAAGTCAAGCTACAAGCTTGCTTACAAAGCTACAAGCCGCGAACGTAAGCCATGAAATATAAT
TATGCTTTTGTATTTTAG

Intron 2: 229bp

GTGAGTTCAAGTACCCGATTAACTCAAATTCCGTCTTAATTTGAAGGAAGTAATTAAAATAATATTTTGTAGTCT
GGGTCTCACTCTTGTTTGTTTTTAAATTATAAAAGCGGAATTTCTTATTCAAATATGTAATTCTGATTCATAAAC
ATTATAAACTTATCTGCTAAGTATATTATAAAATCACGATACTTTATCACATAAAATAAAAATTCATTATTATTT
CCAG

Intron 3: 543bp

GTACTTATAACAGCTGAGTTAAGTCGCATGGGTTTCGACTATATTCACACCACATAGATTCCATAGATTGTAGGT
ATCTACATAATTCCTTTTTTATATTTAAAGCACGCCTACTTTATTTCATCTGTGCACCACCCACTGATAGAACGG
AAAACAAAAAAGTATTATAATTCGCCTATTTTCAATACAAATTCTTAGACCAACTAGAAAAGACTGCAGTGTGCT
TATTGCCGTGAATAAAAAATGACATGATATCTAATCAAATAAATATTAGATATATGTATACAGTGCACTCATAAT
TGTGAGACAGACTTGTCTGTATTTAGACACGTTTAACATTTTTTAACCCCCGATGCAAAGATAGAAGTGTTATAA
GTTTATGGCATGTACCTCTATGTCTGTCTGTCTATGGTATCGTAGCTCCCAAACGAATGGTCGGATTTTGATGCA
ATTATATTTTTTTTCACATATAGATAACGTAAGACATATTCTGTTTATTTTGAAATATAATATTTGTTTACTATG
ATCATTTTTTCCATTTAG
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Fig. 1. Complete sequence of the *Manduca sexta* crustacean cardioactive peptide (CCAP) gene. Nucleotide and predicted peptide sequences are listed for the coding region, which starts at nucleotide 1. Blue, CCAP-encoding region; green, dibasic post-translational cleavage sites; red, glycine used for C-terminal amidation; yellow shading, consensus signal peptide sequence; asterisk, stop codon. Intron position and length are indicated above the nucleotide sequence. Intron sequences are shown separately at the bottom, with underlined GT and AG for the intron donor and acceptor consensus sequences, respectively.

Fig. 2. Expression of the crustacean cardioactive peptide (CCAP) transcript in the brain of *Manduca sexta*. (A) Ventral view of the brain in a day 1 first-instar larva showing a cluster of three anterior pairs of cells (arrows), one anterio-medial pair (concave arrow) and a large ventral pair in the mid protocerebrum (arrowheads). The sixth pair of cells was located on the dorsal side (convex arrow; out of focus). (B) Dorsal view of a brain in a day 2 fourth-instar larva. The 'sixth dorsal pair' of cells is indicated by convex arrows, and the convex arrowheads point to the two pairs of cells that started expressing CCAP after reaching the fourth instar. (C) Posterior view of an adult brain showing two pairs of cells (arrows). Scale bars, 50 μm.

nucleotides long, encoding a predicted precursor protein of 125 amino acid residues, and contains a highly predicted signal peptide sequence with a putative cleavage site between residues 22 and 23 (Nielsen et al., 1997). The single copy of CCAP in the predicted precursor protein is preceded by a KR and followed by an RK doublet of basic amino acid residues that serve as internal proteolytic cleavage sites during post-translational processing of the proprotein (Loh and Gainer, 1983). A glycine residue is encoded between the end of the CCAP-coding region and the beginning of the RK cleavage site. This glycine forms the amidated end of the peptide post-translationally (Fig. 1). No other putative peptides are encoded in the CCAP-coding precursor. There are 750 untranslated base pairs between the stop codon and the beginning of the poly(A) tail. Three introns were also identified. Intron 1 is located before the CCAP-coding region, between residues 24 and 25, and is 243 bp long. Intron 2, 229 bp in length, is found in the glycine residue forming the C-terminal amide (residue 52; phase 1) in the CCAP-coding region. The final intron, intron 3, is 543 bp long and is located between residues 73 and 74, after the CCAP-coding region.

Distribution of cells in Manduca sexta *expressing the CCAP gene*

To determine the expression pattern of the CCAP gene described above throughout post-embryonic life, *in situ* hybridization was performed on the *M. sexta* CNS using a probe from the subcloned PCR product of the CCAPT3 and CCAPT7 primers, which contained the middle (coding) region of the CCAP gene. *In situ* hybridization was carried out on the complete CNS, as described in the Materials and methods section, and was performed at all major

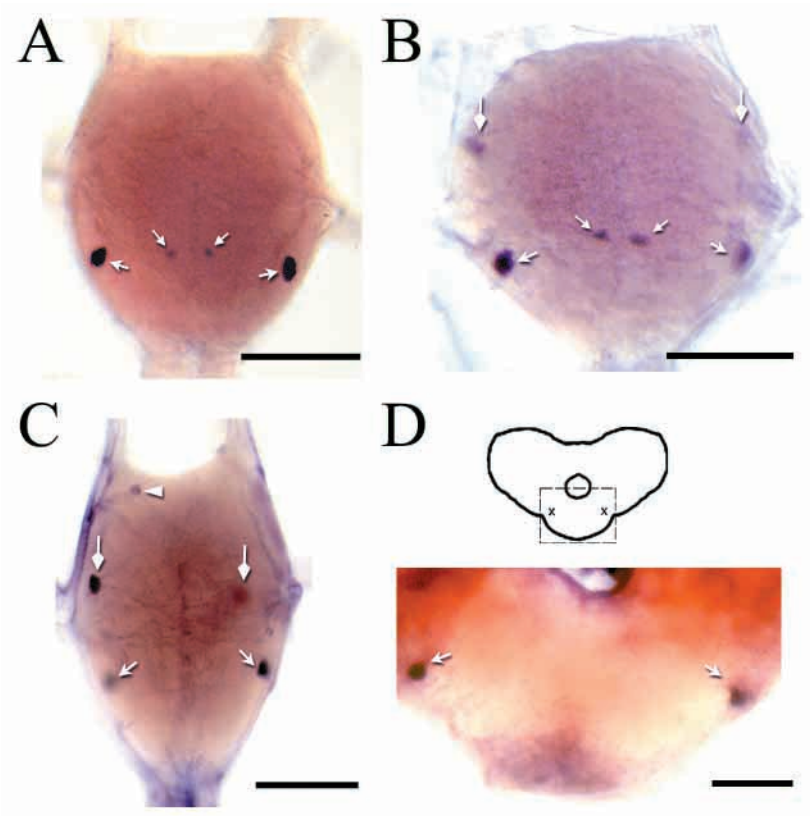

Fig. 3. Expression of the crustacean cardioactive peptide (CCAP) transcript in the subesophageal ganglion (SEG) of *Manduca sexta*. (A) Ventral view of an air-filled third-instar SEG showing a pair of posteriolaterally located cells (concave arrows) and a pair of smaller midline cells (arrows). (B) Ventral view of an air-filled fourth-instar larval SEG showing the previously discussed four cells (described in A) and an anteriolateral pair (convex arrows; left cell out of focus). (C) Ventral view of an SEG from a day 0 fourth-instar larva showing the posteriolaterally located cells (concave arrows), the anteriolateral cells (convex arrows) and a single anterior cell to the left of the midline (arrowhead). (D) Posterior view of an adult SEG showing only one pair of CCAP-expressing cells (arrows). Inset: diagram of the adult brain showing the area from where D was obtained (box; X indicates positions of cells). Scale bars, 50 μm.

developmental stages: L1 (first-instar larva), L2, L3, L4, L5 D0 (day 0, fifth instar), L5 W0 (wandering day 0, fifth instar), L5 W3 (wandering day 3, fifth instar), P0 (pupal day 0), P10 (pupal day 10, midway through adult development), pharate adult (the day of adult emergence prior to ecdysis) and adult day 0 (A0; the day of adult emergence after ecdysis and wing inflation have been fully completed). During analysis of these data, several major points emerged. CCAP-labeled cells were found in every stage of development and in nearly all CNS ganglia. CCAP gene expression levels in some cells were very robust, remaining high at every stage from the first instar to adult emergence.

In other cells, CCAP gene expression levels fluctuated greatly over the course of several developmental stages (e.g. over 2–3 instars). Some cells did not express the CCAP gene until later in development. The appearance of these late-expressing cells resulted in an overall increase in the number of CCAP-expressing cells throughout the larval stage, especially in the late fourth-instar, fifth-instar and early pupal (P1–P4) stages. The highest number of cells expressing the CCAP gene occurred at the larval–pupal transition. Following this peak, there was a drop in cell number midway through adult development (P10). The number and pattern of cells expressing the CCAP gene at P10 were similar to those in newly emerged adults, except for a few midline terminal ganglion cells that lost CCAP gene expression at the pharate adult stage. Although nearly all cells have a counterpart in the contralateral hemi-ganglion, sometimes only one of the pair expressed the CCAP gene. This occurred mostly with the abdominal midline cells and with a cell in the anterior-most part of the subesophageal ganglion (SEG). The following sections describe the spatial and temporal expression of CCAP gene expression in each ganglion and address each of the above points in detail.

Brain

In early larval stages (first- to third-instar larvae), six pairs of cells were labeled by the *in situ* probe. Four pairs were located in the anterior part of the brain. Three of them formed a cluster, and the fourth was slightly medial to that cluster (Fig. 2A). The fifth pair was located on the ventral side of the mid protocerebrum and was larger, approximately twice the size of the other cells (Fig. 2A). A sixth pair of cells was located on the dorsal side of the mid protocerebrum. After D2 of the fourth instar, two more pairs of cells expressing the CCAP gene were added to the dorsal part of the brain (Fig. 2B). Another pair of ventrally situated cells, located near the larger pair of cells described above, started expressing the CCAP gene intermittently at D3 fifth instar and only consistently after W0 fifth instar (not shown). All positively labeled cells in early instars (first–third) continued to express the CCAP gene until wandering. By W3, only three pairs of cells, two dorsal and one ventral, remained expressing the CCAP gene. After adult emergence (A0), only two pairs of cells were observed, but their relationship to the larval brain cells described above is unknown (Fig. 2C).

Subesophageal ganglion (SEG)

The SEG of first- to third-instar larvae contained two pairs of cells that labeled with the CCAP *in situ* probe. One pair was located in the posteriolateral part of the ganglion, and the other pair was situated along the midline (Fig. 3A). Detectable CCAP gene expression in the lateral cells persisted throughout all post-embryonic stages, in contrast to that in the midline cells whose staining disappeared entirely at pupation. At the beginning of the fourth instar, a third pair of cells was observed adjacent to the lateral cells. The level of CCAP gene expression in these cells fluctuated slightly from D0 fourth instar until the early pupal stage (results not shown). Because of the reorganization of the SEG during adult development, it was not clear whether these cells continued to express the CCAP gene towards the end of adult development.

A fourth pair of *in situ* stained cells, located anteriorly to the laterally located pairs, was observed starting in a few animals in the head capsule phase late in the third instar (immediately prior to molting) or in approximately 10 % of D2 fourth-instar animals (Fig. 3B). Positive staining in these cells, in terms of percentage and intensity, increased throughout the fourth instar until approximately 80 % of observed cells were stained at the air-filled head capsule stage of the fourth instar (Copenhaver and Truman, 1982). CCAP gene expression in these cells varied throughout the fifth instar, but became consistent after W0. It was not possible to ascertain conclusively expression levels in this pair of cells in post-larval stages because of the major reorganization of the SEG during this period.

Another cell was also stained using the CCAP probe. This cell was located at the anterior-most part of the ganglion (Fig. 3C). Staining of this cell was weak and was observed mostly in fourth and feeding fifth instars. Although this cell seemed to be one of a pair, only the left cell (ventral view) ever expressed this message. In early pupae, only two pairs of cells expressed the CCAP gene (data not shown), and at A0 only one laterally situated pair remained (Fig. 3D).

Thoracic ganglia (T1–T3)

The overall pattern of CCAP gene expression was essentially the same in all three thoracic ganglia; three pairs of cells were labeled in each ganglion, two of which were adjacent to each other and located laterally. A third pair was situated anteriorly and slightly medially to the others (Fig. 4B). In the head capsule phase (HCP) of third-instar larvae, expression of the CCAP gene began to appear in the larger of the two laterally located pairs in all three thoracic ganglia (Fig. 4A). However, expression was not consistent in all preparations. It fluctuated between no expression, moderate expression and high expression in the same stage (Fig. 5A–D). The CCAP gene in the larger lateral cells was expressed in 20 % of feeding third-instar larvae, 70 % of feeding fourth-instar larvae and approximately 70 % of feeding fifth-instar larvae and reached 100 % at wandering. Interestingly, these cells were almost always transiently labeled by the probe at the fourth-instar HCP stage (10 out of 11 trials). These large lateral cells were also stained in all post-larval stages (Fig. 4C–F).

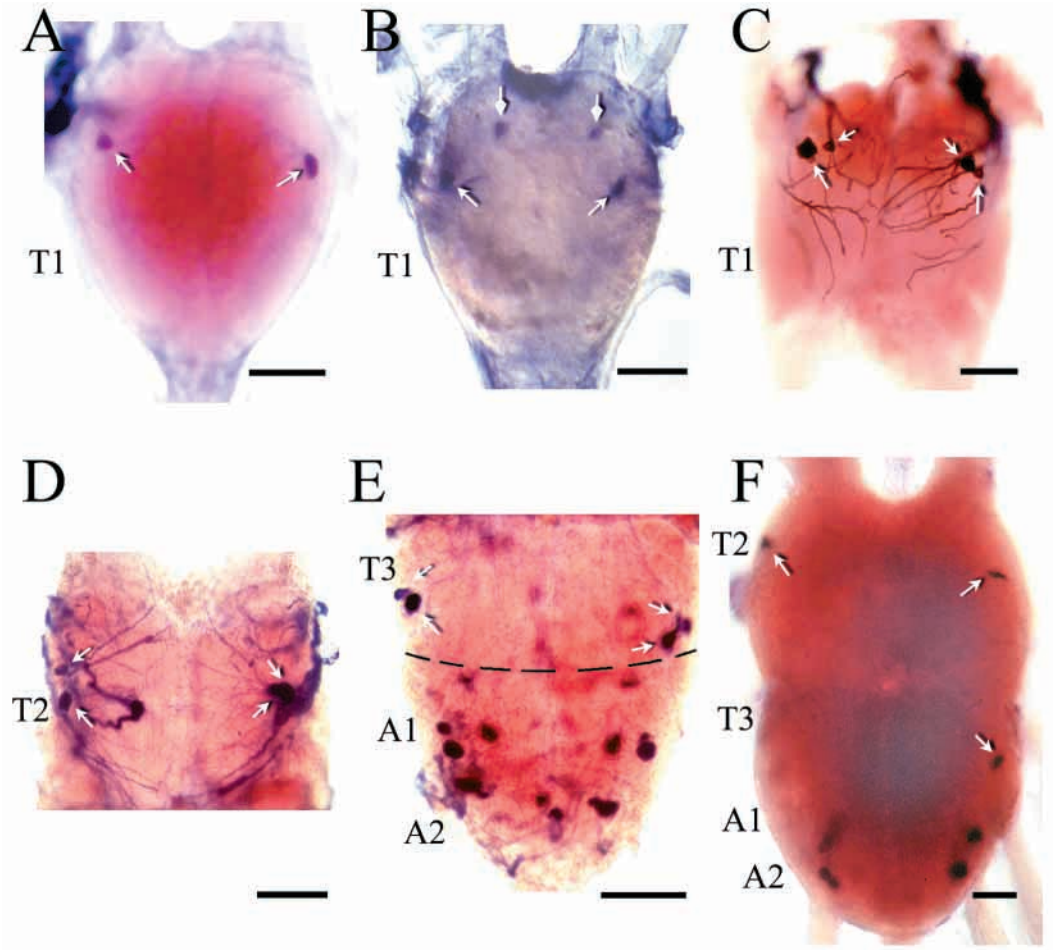

Fig. 4. Expression of the crustacean cardioactive peptide (CCAP) transcript in the thoracic ganglia (T1–T3) of *Manduca sexta*. (A) Ventral view of T1 from a day 0 fourth-instar larva showing a pair of lateral cells (arrows) labeled by the *in situ* hybridization probe for CCAP. (B) Ventral view of a wandering day 3 fifth-instar T1 showing a pair of labeled lateral cells (concave arrows) and a second pair of more anterior cells (convex arrows). (C) Ventral view of T1 from a day 10 pupa (P10) showing two pairs of lateral cells (concave arrows). (D) The anterior portion (T2) of the fused thoracic ganglion of a P10 pupa. The concave arrows point to two pairs of lateral cells. (E) The posterior portion of the fused thoracic ganglion in a P10 pupa with two pairs of lateral cells (concave arrows) in the T3 neuromere. The dotted line marks the approximate boundary between T3 and A1 neuromeres. (F) The fused thoracic ganglion of a day 0 adult. The lateral cells in T2 and T3 are indicated by concave arrows. Scale bars, 50 μm.

The other two pairs of thoracic neurons started expressing the CCAP gene at different stages. The smaller lateral neurons, adjacent to the above-mentioned large lateral cells, began expressing the CCAP gene after pupation. CCAP gene expression levels remained high in these cells through P10 but disappeared by A0 (Fig. 4C–F). The third pair of CCAP-expressing cells, located in the anterior part of the thoracic ganglia (Fig. 4B), expressed the CCAP gene at different stages in different ganglia. The anterior cells in the metathoracic ganglion (T3) were the first to express the CCAP message, beginning in the late feeding fifth-instar (D3 fifth) stage. In the mesothoracic ganglion (T2), the anterior pair of cells begin expressing this gene consistently only after wandering (W0). The homologs of these anterior cells in the prothoracic ganglion were labeled weakly and briefly beginning in some late wandering fifth instars (30 %) and early pupae (30 %). CCAP gene expression had ceased in all anterior cells in all thoracic ganglia by P10.

Abdominal ganglia (A1–A6)

The general pattern of expression was, with some minor differences, the same for all unfused abdominal ganglia and is reminiscent of the expression pattern in the thoracic ganglia: two pairs of posteriolaterally situated cells, a pair of anteriolateral cells and several unpaired midline cells. In every abdominal ganglion at every stage (first instar to A0), a pair of cells located in the lateral part of the ganglion was consistently and robustly labeled with the probe (Fig. 6A–G). A second pair, adjacent to this robust pair, was also labeled, but the level of CCAP gene expression varied throughout all larval stages and differed slightly between ganglia (Fig. 6C). These cells were irregularly present after pupation, but appear to cease expression of the CCAP message by P10 and did not regain

expression in the adult (Fig. 6F,G). A third pair, located anteriolaterally, also expressed the CCAP gene beginning in the first instar in A3–A6 (Fig. 6B,H). The A2 anteriolateral cells only started to express the CCAP gene in late third instars, and the A1 homologs exhibited staining beginning at the head capsule phase late in the fourth instar until the adult stage (results not shown). A fourth pair of cells, located posteriorly near the ganglionic midline, began expressing a CCAP-coding transcript in feeding (D3) fifth-instar larvae (Fig. 6C). This expression, however, was not consistent within the same nerve cord. Some ganglia contained two labeled cells, while others had only one. In A1, midline cells were not detected using the CCAP probe. Adult expression was observed only in the midline cells of A2–A5 (Fig. 6G,H). In A6, expression ceased by P10. One ventral unpaired median (VUM) neuron started expressing the CCAP gene in A2–A6 from early pupa to adult (Fig. 6D–F,H–I). A second VUM cell also began expressing this gene midway through adult development (P10; Fig. 6F). At A0, these VUM cells were occasionally observed at a lower intensity in the fused thoracic/abdominal ganglion (not shown) and in A3–A5 (Fig. 6H,I).

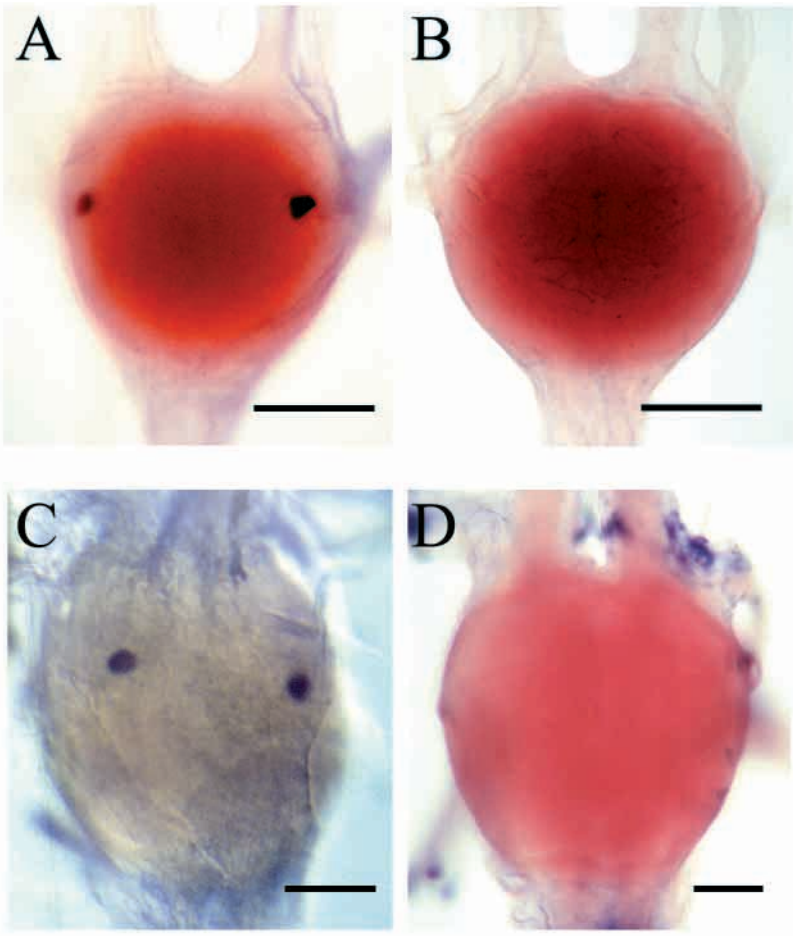

Fig. 5. Variable nature of crustacean cardioactive peptide (CCAP) transcript expression in a bilateral pair of lateral neurosecretory cells, NS-L(T), in the thoracic ganglion of *Manduca sexta*. (A) The lateral cells in T3 of a third-instar larva. (B) T3 of another third-instar larva with no labeled cells. (C) The lateral cells in T1 of a day 3 fifth-instar larva with two cells labeled. (D) T1 of another day 3 fifth-instar larva with no labeled cells. Scale bars, 50 μm.

Terminal ganglion (TG)

The two-lobed larval TG is a fusion of ganglia serving segments A7–A10. The anterior neuromere of TG, referred to as neuromere 7, serves segment A7. The posterior neuromere serves segments 8, 9 and 10 and will be referred to as the terminal neuromere. From first- to third-instar larvae, only two pairs of cells were labeled (Fig. 7A). These four cells were located in the lateral part of neuromere 7. In late fourth-instar (HCP fourth) larvae, two more pairs of cells in the terminal neuromere of the TG were labeled (Fig. 7B,C). The level of gene expression in these two pairs of cells fluctuated until wandering, at which time staining was more consistent and present until A0. Occasionally, there were two additional pairs of cells (Fig. 7D, convex arrowheads) in the terminal neuromere that began CCAP gene expression in D3 fifth-instar larvae. These two pairs expressed the transcript until the early pupal stage (P1–P3), at which time CCAP gene expression disappeared completely from these cells. A seventh stained pair, located in the most anterior part of neuromere 7, was transiently observed in HCP third-instar and HCP fourth-instar larvae. Staining in these cells stabilized in late feeding fifth-instar larvae (D3) and persisted until P10 (Fig. 7C,D). A single unpaired neuron in neuromere 7, approximately the size and shape of an abdominal ganglion VUM cell, began to be labeled after wandering, appearing in approximately 10 % of the preparations. By P10, this cell was more frequently stained; it was observed in approximately 60 % of our preparations (Fig. 7E). By A0, this VUM-like cell and its A6 counterpart had both stopped expressing the CCAP gene (Fig. 7F). A0 adults have several sets of cells expressing the CCAP gene distributed throughout the TG (Fig. 7F), but their relationship to their larval counterparts was not possible to ascertain because of the major reorganization of the terminal ganglion during metamorphosis.

Summary of the expression pattern of the CCAP gene in the Manduca sexta *CNS*

The temporal and spatial expression patterns of the CCAP gene in the *M. sexta* CNS are summarized in Fig. 8. The CCAP gene is expressed in a total of 116 neurons in the post-embryonic *M. sexta* CNS, counting any cell marked by the *in situ* probe in any developmental stage from first-instar larva to adult day 0. Nine pairs of cells were observed in the brain, 4.5 pairs in the SEG, three pairs in each thoracic ganglion (T1–T3), three pairs in A1, five pairs each in A2–A6 and 7.5 pairs in the terminal ganglion. The CCAP gene is expressed

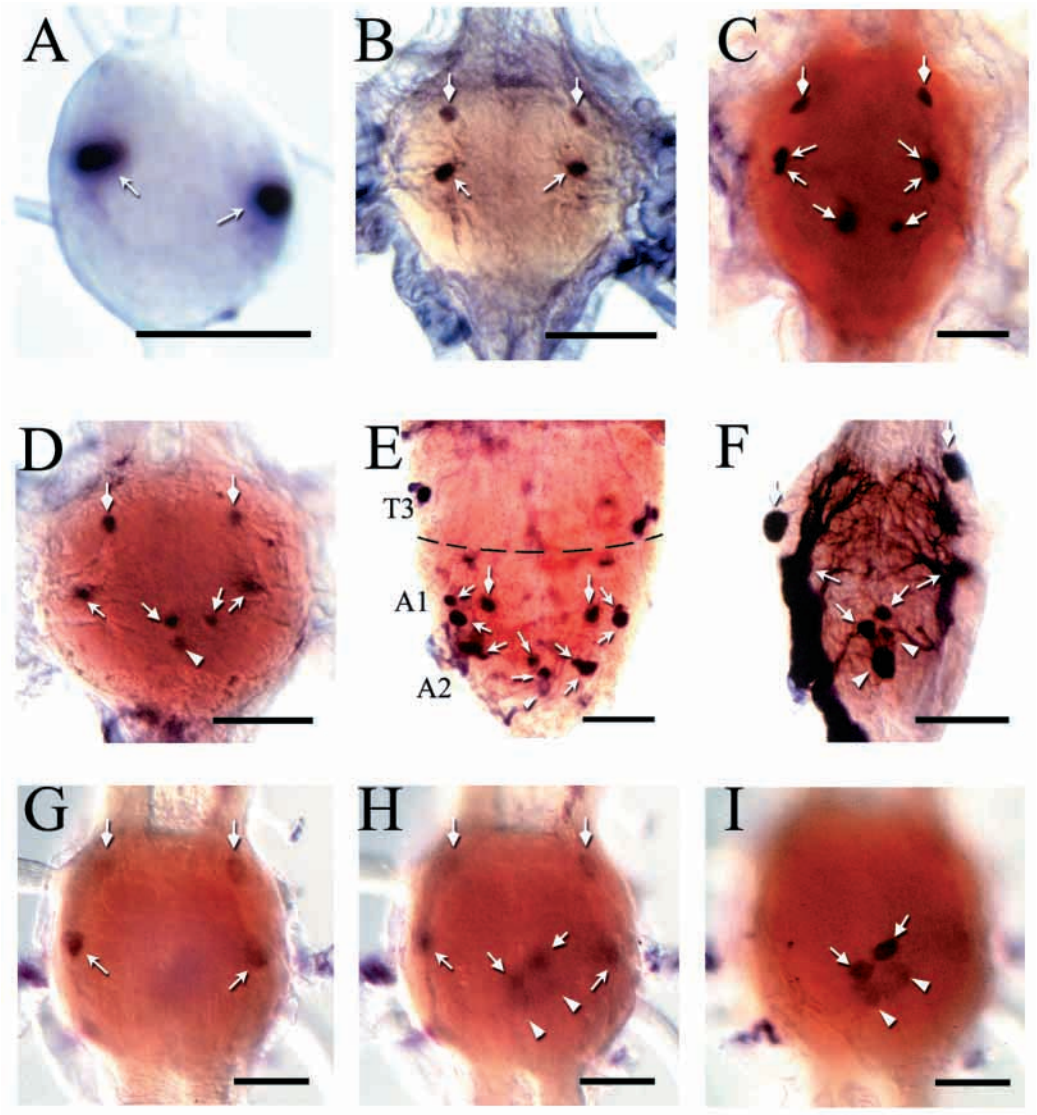

Fig. 6. Expression of the crustacean cardioactive peptide (CCAP) transcript in the abdominal ganglia (A1–A6) of *Manduca sexta*. (A) Cells labeled with the *in situ* probe in the first abdominal ganglion (A1) from a day 2 second-instar larva. (B) Labeled cells in A2 of a day 1 fifth-instar larva. (C) Labeled cells in A2 of a day 3 fifth-instar larva. (D) Labeled cells in A4 of a day 0 pupa (P0). (E) Labeled cells in the posterior portion (T3, A1 and A2) of the fused thoracic ganglion from a day 10 pupa (P10). (F) Labeled cells in A3 of a P10 pupa. (G–I) Three different planes of focus of A3 in a day 0 adult, from ventral (G) to dorsal (I). (A–I) Lateral cells, concave arrows; anterior cells, convex arrows; midline cells, arrows; ventral unpaired neurons, arrowheads. Scale bars, 50 μm.

in every ganglion in each post-embryonic stage (Fig. 8, Fig. 9), except in the thoracic ganglia of first- and second-instar larvae. In total, 52 cells express the CCAP gene in first-instar larvae (Fig. 9). This number increases steadily throughout larval life, with additional cells observed in all CNS regions, and reaches a peak of 116 cells shortly after pupation. Following this peak, the total number of cells in the CNS expressing the CCAP gene declines through the rest of adult development, with 87 remaining at the pharate adult stage.

Discussion

CCAP gene structure in Manduca sexta

The molecular data in this study provide the first detailed glimpse of the structure of the CCAP gene in any organism. The *M. sexta* CCAP gene encodes a single copy of CCAP and no other peptides, a somewhat surprising result considering that many peptide genes have multiple copies of the same small peptide (e.g. 11 copies of FMRFa in the cuttlefish FMRF gene; Loi and Tublitz, 1997) and/or often code for multiple peptides (e.g. 11 different FMRFa peptides in the *D. melanogaster* FMRFa gene; Schneider and Taghert, 1988). Another unusual aspect of the CCAP gene is the presence of an intron (intron 2) in the middle of the peptide-coding region (Fig. 1). In this instance, the intron is located in the C-terminal residue (glycine). A similarly located intron is thought to be present in the *D. melanogaster* CCAP gene (R. Jackson, personal communication; see below). Introns in peptide-coding regions are relatively rare; examples in insects are the introns found in the PBAN-coding genes in the corn earworm *Helicoverpa zea* (Davis et al., 1992) and in the silkworm *Bombyx mori* (Xu et al., 1995). The underlying rationale for the presence of an intron in the coding region of a peptide gene awaits further analysis.

Comparison of the Manduca sexta *and* Drosophila melanogaster *CCAP gene structures*

A BLAST search of the *Drosophila* genome database for sequences similar to the *Manduca* CCAP gene revealed a

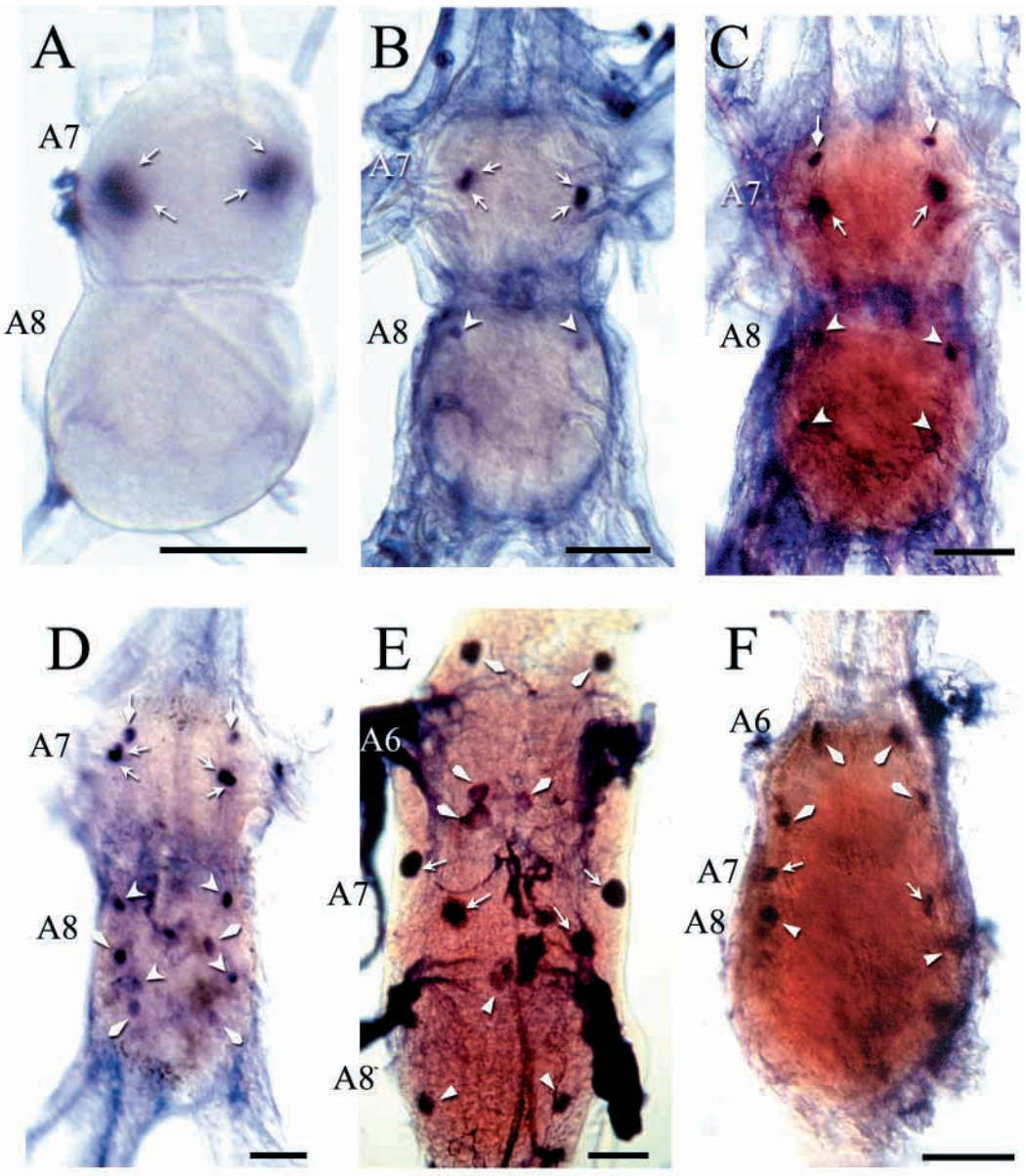

Fig. 7. Expression of the crustacean cardioactive peptide (CCAP) transcript in the terminal ganglion of *Manduca sexta*. (A) Day 2 second-instar larva. (B) Air-filled fourth-instar larva. (C) Day 3 fifth-instar larva. (D) Wandering day 3 fifth-instar larva. (E) Day 10 pupa. (F) Day 0 adult. (A–D) Terminal ganglia of larvae are formed by the fusion of the seventh abdominal ganglion (denoted as A7) and abdominal ganglia 8–10 (denoted as A8). Lateral cells, concave arrows; anterior cells, convex arrows; A8 cells started to express the CCAP transcript after the head capsule stage of a fourth-instar larva, concave arrowheads; two pairs of cells that express the CCAP transcript after wandering, convex arrowheads. (E,F) Terminal ganglia of late pupae (pupal day 14) and adults (adult day 0) are formed by the fusion of the sixth abdominal ganglion (denoted A6) with A7 and A8–A10. CCAP-labeled cells in A6, convex arrowheads; in A7 cells, concave arrows; in A8–A10 cells, arrowheads. Scale bars, 50 μm.

single matching region annotated as CG4910. Using these data plus RT-PCR and RACE methods, we found that the structure and coding sequence of the *D. melanogaster* CCAP gene are very similar to those of the *M. sexta* CCAP gene. The *M. sexta* and *D. melanogaster* CCAP genes each encode a single copy of CCAP and no other peptide (Fig. 10). Amino acid sequence alignment between *M. sexta* and *D. melanogaster* CCAP genes, using blosum62 with gap penalty 10 and extension penalty 0.1 (Henikoff and Henikoff, 1993), found significant homology between the two CCAP genes (46.2 % identity and 58 % similarity; Fig. 10). The *D. melanogaster* CCAP gene, compared with the *M. sexta* CCAP gene, contains a similar number of untranslated nucleotides at the 5′ end (77 *versus* 66) but a significantly smaller number at the 3′ end (178 *versus* 750). Another interesting similarity between the *D. melanogaster* and *M. sexta* CCAP genes is the presence of the second intron located in both genes in an identical position (glycine residue) and phase (phase 1) within the region coding for the CCAP peptide (data not shown). Given the organizational and structural similarities between the *D. melanogaster* and *M. sexta* CCAP genes, it is likely that these two genes arose from a common ancestral gene. A complete sequence analysis of this *D. melanogaster* CCAP gene has recently been reported by R. Jackson (personal communication).

The CCAP gene is probably responsible for most if not all CCAP expression in the M. sexta *CNS*

If the CCAP-encoding gene described in these studies is responsible for the production of all CCAP in the *M. sexta* CNS, then there should be a high degree of concordance in the expression patterns of the CCAP gene and CCAP peptide. To assess the validity of this hypothesis, we compared our *in situ* data with CCAP immunocytochemical results consolidated

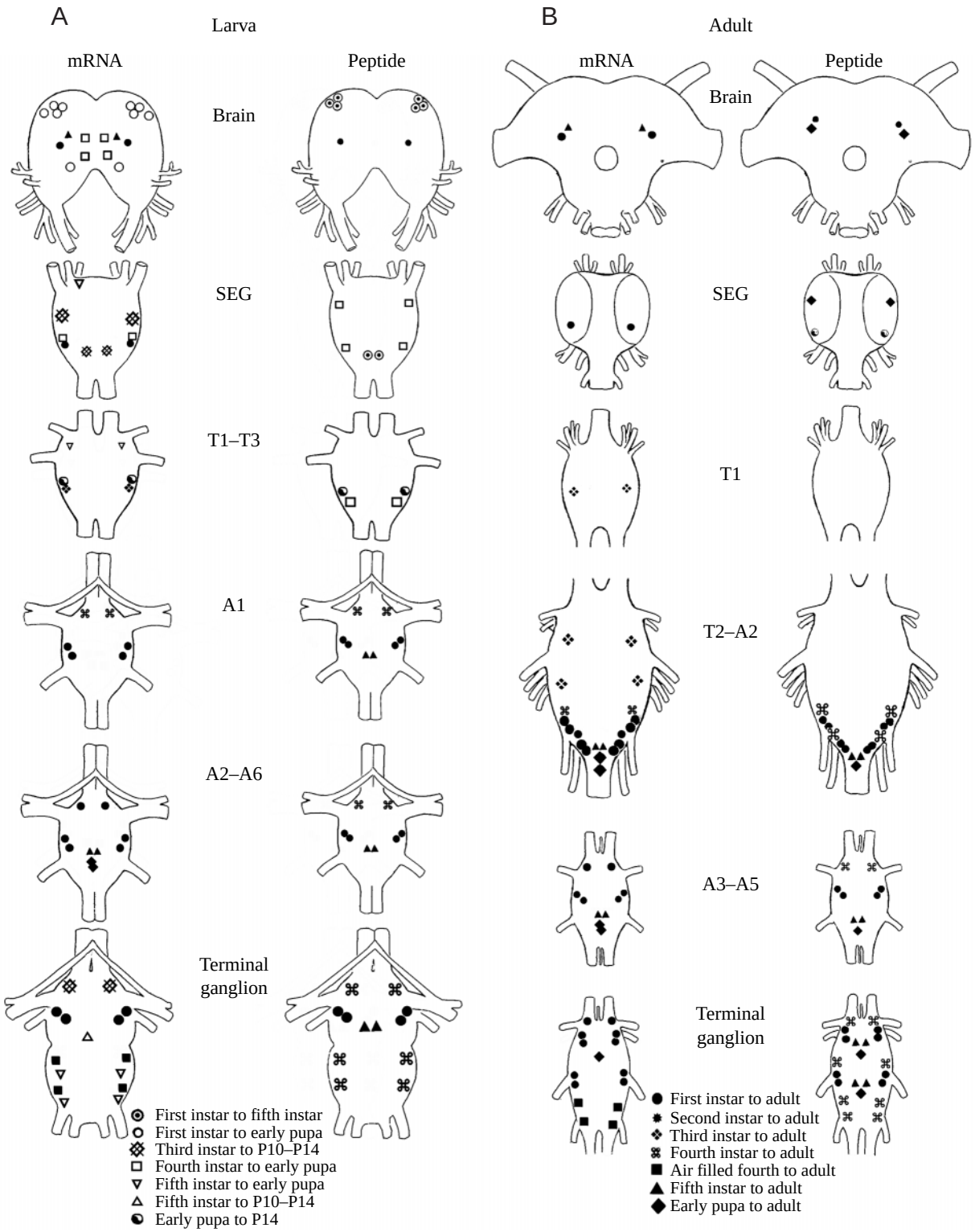

Fig. 8. Summary of all cells in the larval and adult *Manduca sexta* central nervous system (CNS) labeled with the *in situ* probes and crustacean cardioactive peptide (CCAP) antibodies. (A) CNS cells in *Manduca sexta* larvae expressing CCAP mRNA (left) and exhibiting CCAP-like immunoreactivity (right). (B) Cells in adult *Manduca sexta* CNS expressing CCAP mRNA (left) and exhibiting CCAP-like immunoreactivity (right). Antibody data are taken from the results of Davis et al. (Davis et al., 1993) and Klukas et al. (Klukas et al., 1996).

from published reports of CCAP antibody staining in the *M. sexta* CNS (Davis et al., 1993; Klukas et al., 1996; Ewer and Truman, 1996). On the basis of CNS location, cell size and developmental information, we determined that 91 of 95 CCAP-like immunoreactive cells observed in the CCAP antibody staining studies were also labeled by the *in situ* probe.

Table 1. *Identification of the 91 cells in the* Manduca sexta *central nervous system that co-label for the crustacean cardioactive peptide (CCAP) transcript and the anti-CCAP antibody*

	Cells expressing CCAP mRNA	Cells stained with anti-CCAP antibody	Abbreviation
Brain	Three pairs of cells in the anterior part of the brain	Interneurons of the anterior dorsal protocerebrum	IN-AD
	One large pair of cells in the mid protocerebrum	Interneurons of the mid protocerebrum	IN-MP
Sub-esophageal ganglion (SEG)	One pair of anteriorly located cells	Labial neurosecretory cells	NS-L(Mx)
	One pair of laterally located cells	Maxillary neurosecretory cells	NS-L(Mx)
	One pair of midline cells	Ventral median interneurons of the SEG	IN-VM
Thoracic ganglia (T1–T3)	One pair of laterally located cells	Lateral neurosecretory cells of thoracic ganglia	NS-L(T)
	Second pair of laterally located cells adjacent to the NS-L(T)	Interneurons 704 of thoracic ganglia	IN-704(T)
Abdominal ganglia (A1–A6)	One pair of anteriorly located cells	Motoneuron 1 of abdominal ganglia	MN-1
	One pair of laterally located cells	Lateral neurosecretory cells of abdominal ganglia	NS-L
	Second pair of laterally located cells adjacent to the NS-L	Interneurons 704 of abdominal ganglia	IN-704
	One pair of cells located in the midline	Paired median neurosecretory cells of abdominal ganglia	NS-M
	One large unpaired cell in the midline	The ventral unpaired median neuron	VUM
Terminal ganglion	One pair of anteriorly located cells in neuromere 7	Motoneuron 1 of neuromere 7	MN-1
	One pair of laterally located cells	Lateral neurosecretory cells of neuromere 7	NS-L
	Second pair of laterally located cells adjacent to the NS-L	Interneurons 704 of neuromere 7	IN-704
	One large unpaired cell in the midline	The ventral unpaired median neuron	VUM
	Two pairs of anteriorly located cells in the terminal neuromere	Motoneuron 1 of terminal neuromeres	MN-1

Antibody data are taken from Davis et al. (Davis et al., 1993) and Klukas et al. (Klukas et al., 1996).

We compared the location of the labeled cells in the present study with the descriptions of the location of the cells immunostained with CCAP antibodies (Davis et al., 1993; Klukas et al., 1996; Ewer and Truman, 1996) and were able to identify 91 of 116 cells labeled with the *in situ* probe. Table 1 lists all 91 cells from the *in situ* hybridization experiment that have a counterpart in the CCAP antibody staining data. For example, three of the four pairs of *in-situ*-labeled cells located in the anterior part of the brain correspond to the CCAP-immunopositive cells called interneurons of the anterior dorsal protocerebrum (IN-AD) in the study of Davis et al. (Davis et al., 1993). Similarly, the pair of lateral cells in every ganglion in the ventral nerve cord that express both the CCAP gene and peptide are likely to be one of the lateral neurosecretory cells (NS-L) and interneuron 704 (IN-704).

In addition to the 91 cells that express the CCAP gene and peptide, 25 cells expressed the CCAP gene but were not stained with the CCAP antibody. The absence of peptide expression in the 25 cells exhibiting CCAP gene activity suggests three possibilities: (i) that these cells do not translate the transcript into peptides; (ii) that the amount expressed is below the antibody detection threshold, or (iii) that peptide expression is very transient. This study did not distinguish between these possibilities.

There are also four CCAP-immunoreactive neurons that did not express the CCAP gene. These are a pair of neurons in larvae with midline cell bodies located in the posterior region

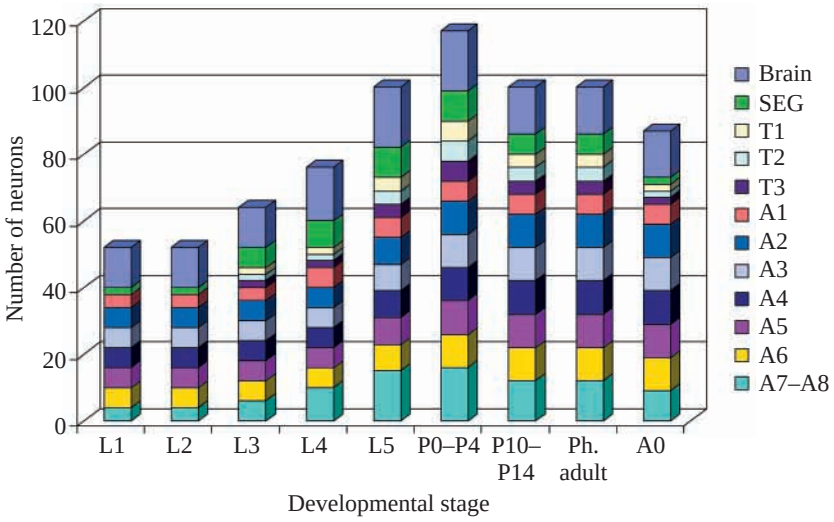

Fig. 9. Temporal expression of the crustacean cardioactive peptide (CCAP) transcript in the *Manduca sexta* central nervous system. Each central nervous system region is color-coded. A, adult; L, larva; P, pupa; Ph. Adult, pharate adult; SEG, subesophageal ganglion; T, thoracic ganglion.

of the first abdominal ganglion and their homologs in the posterior neuromere of the adult terminal ganglion (Fig. 8). The inability to detect CCAP gene activity in these four cells may be the result either of rapid degradation of CCAP mRNA in these cells or artifactual CCAP antibody staining. Future *in situ* and antibody analyses of these four cells should distinguish between these possibilities.

These data taken together suggest that the CCAP gene isolated in the present study is the major and perhaps only CCAP gene in the *M. sexta* CNS. Of the 95 known CCAP-immunoreactive cells, 91 also expressed the CCAP gene. This leaves open the possibility either that the CCAP gene described in this paper produces more than one CCAP-coding transcript or that there is a second CCAP-coding gene in *M. sexta*. If a second CCAP transcript or gene exists in *M. sexta*, its contribution to overall CCAP expression in the *M. sexta* CNS is very minimal. To date, we have no evidence to support the alternative transcript hypothesis in *M. sexta*, and the *D. melanogaster* genome annotations predict only one transcript from the region associated with CG4910. There is also no molecular or bioinformatic evidence suggesting the presence of a second CCAP gene in *M. sexta* or *D. melanogaster*. Hence, we believe that the CCAP gene identified here is likely to be the only CCAP-coding gene in *M. sexta* and probably also in *D. melanogaster*.

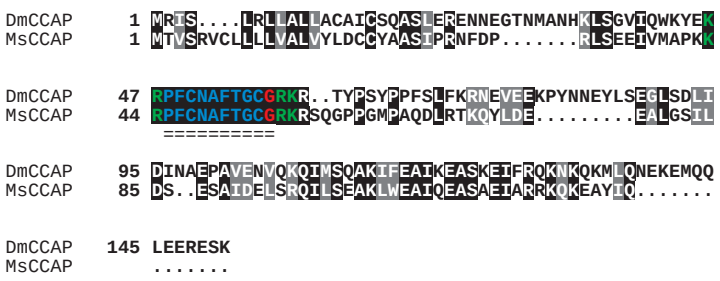

Fig. 10. Alignment and comparison of deduced amino acid sequences of crustacean cardioactive peptide (CCAP)-encoding mRNA from *Drosophila melanogaster* (DmCCAP) and *Manduca sexta* (MsCCAP). The CCAP-coding region: blue; C-terminal glycine, red; putative post-translational cleavage sites, green. Sequence alignment was obtained using blosum62 with gap penalty 10 and extension penalty 0.1. Periods (.) are gaps generated for the alignment. Identical and similar amino acids are in black and gray shaded boxes, respectively

CCAP gene expression and plasticity in NS-L(T) and IN-704(T)

Previous studies using anti-CCAP antibody produced conflicting results as to whether NS-L(T) and IN-704(T) in the thoracic ganglia, the small and large lateral cells described in our results, contain CCAP (Klukas et al., 1996; Davis et al., 1993). One study (Klukas et al., 1996) provided positive evidence in support of this assertion, while another (Davis et al., 1993) found no immunostaining in these cells. Our data demonstrated that IN-704(T), the smaller lateral cell, expresses the CCAP gene starting after pupation up to and including the pharate adult stage (Fig. 8). NS-L(T), the larger cell body, exhibits an unusual degree of plasticity in the expression of the CCAP gene. The CCAP gene is variably expressed in NS-L(T) from third-instar larvae to A0. CCAP gene expression levels in NS-L(T) appeared to be highly variable throughout this period, much more so than in other neurons. Interestingly, gene expression was not always consistent between different individuals at the same stage of development. We observed the highest variability in CCAP gene expression at the HCP stage in fourth-instar larvae, which precedes the beginning of overt ecdysis behavior, to the fifth instar (Fig. 5). At this stage, CCAP gene expression is observed in some but not every pair of NS-L(T) cells. This variability is unlikely to be due to poor penetration by the probe in some preparations since all preparations exhibited *in-situ*-labeled cells. These results suggest two, non-exclusive possibilities: (i) that CCAP gene expression in NS-L(T) is very transient at the HCP stage and/or (ii) that, in some preparations, NS-L(T) at the HCP stage does not generate the CCAP transcript. It has been hypothesized that NS-L and IN-704 in each ventral ganglion are involved in initiating ecdysis behavior through the release of CCAP (Ewer and Truman, 1996; Ewer and Truman, 1997). If NS-L(T) and IN-704(T) are involved in the ecdysis circuit, then the release of CCAP might trigger a new round of *de novo* CCAP synthesis. Alternatively, these thoracic cells might not be directly involved in ecdysis behavior, hence the uncoordinated expression pattern observed in different preparations. A complete understanding of the role of these cells in ecdysis behavior awaits further research.

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